Morphology of Filaments on the Chorion of Oocytes and Eggs in the Medaka

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ABSTRACT—The morphology of filaments on the chorion of oocytes and eggs of the medaka, *Oryzias latipes*, was investigated as a step in the clarification of the mechanism for the determination of egg polarity. In mature oocytes, long attaching filaments arising from the vegetal pole area (VPA) of the egg membrane (chorion) were wound spirally on the axis between the animal and the vegetal poles of the egg, and covered the chorionic surface in the vegetal hemisphere. Short non-attaching filaments which were distributed on the chorionic surface in the area other than the VPA were also bent in a unilateral direction on the egg axis. In the previtellogenic stage of oogenesis, both attaching and non-attaching filaments appeared as verruciform structures on the oocyte surface before appearance of the chorion. The VPA at the one end of the animal-vegetal axis was first recognizable morphologically by the appearance of a cluster of verruciform structures which were destined to develop into attaching filaments. As these primitive filaments grew to form horn-shaped structures, their distal parts bent and pointed either to the right or the left around the axis. Six inbreedings of fish selected for right-handed filament pattern failed to demonstrate a hereditory determination of the pattern. In addition, it was ascertained that eggs of several other species of *Oryzias* also possessed both attaching and non-attaching filaments on the chorionic surface which showed an unilateral spiral pattern on the animal-vegetal axis. The results of this study suggest that in the medaka, egg polarity is established by localization of the VPA, followed by the unilateral bending of non-attaching filaments on the chorion.

INTRODUCTION

In teleostean fishes, the oocyte surrounded by developing follicular cells differentiates and grows to form a large telolecithal egg. This specialized cell has a thick extracellular envelope (chorion) resembling the cell wall of a plant cell. Young oocytes prior to formation of the chorion are driven to differentiate into eggs as a result of their interaction with the surrounding granulosa cells. The heterogeneous differentiation of the follicular cells and the formation of unequal structures in the chorion determine egg polarity, i.e. the regional differences in intracellular structures and reactivity of the egg itself. The micropylar cell which forms a pore (micropyle) in the chorion differentiates from granulosa cells [1]. The side of the oocyte where the micropylar cell is located becomes the animal pole of the egg. On the other hand, hair-like structures are located on the chorionic surface at

the pole (vegetal pole) opposite to that of the micropylar cell. These structures are termed "attaching filaments" in *Oryzias* [2–5] and chorionic fibrils in *Fundulus* [6]. The attaching filaments differentiate in the previtellogenic stage of oogenesis in *Oryzias* [7]. The egg axis between the animal and the vegetal poles is easily distinguishable due to these morphological characteristics of the chorion, as well as to the unequal distribution of ooplasmic inclusions. The *Oryzias* egg seems to be a suitable model for clarification of the mechanism by which egg polarity is determined during oogenesis in the teleost.

The main purpose of the present study was to obtain basic information on the dynamic relationship between the oocyte and its surrounding follicular cells, because this information is most likely to clarify the mechanism of determination of egg polarity. It has previously been reported that attaching filaments extend in a spiral pattern from the animal-vegetal axis and cover the chorionic surface in the vegetal hemisphere of the *Oryzias* egg [8]. In a single batch of eggs, eggs bearing

filaments wiht both left- and right-handed spirals can be found. In connection with the spiral pattern of the attaching filaments, the present author is interested in (a) the process by which the egg axis is determined and the spiral pattern of the chorionic filaments is formed during oogenesis, (b) whether or not the spiral pattern of attaching filaments is inherited, and (c) whether or not the spiral pattern is common in this genus. The present study was performed to clarify these points. The results indicate that (a) the spiral pattern around the animal-vegetal axis of the oocyte is first recognized in non-attaching filaments on the oocyte in the early previtellogenic stage of oogenesis, (b) the pattern is probably not inherited, and (c) in all species of *Oryzias* examined, the attaching filaments are arranged in a left- or right-handed spiral pattern.

MATERIALS AND METHODS

Most of the medakas used in the present study were *Oryzias latipes* (Yamato-koriyama, Nara Pref.). In order to compare egg of *O. latipes* with those of other species of *Oryzias,* spawned eggs of *O. celebensis, O. curvinotus, O. javanicus, O. mekongensis, O. melastigma* and *O. minutillus,* which had been breeding in our laboratory, were also examined to determine the spiral pattern or the distribution of attaching filaments.

A light-microscopic survey of the spiral patterns of the attaching filaments and non-attaching filaments was conducted on living eggs (oocytes) in each of ovaries using both a binocular dissecting microscope ($\times 20$) and an ordinary light microscope ($\times 100$, Olympus). In cases in which it was difficult to determine the spiral pattern of the attaching filaments, the direction in which the tops of the non-attaching filaments bent was used to make a determination. Fertilized eggs with the right-handed spiral pattern were selected and allowed to develop to fry. Newly hatched fry were raised to adults in a glass bowl (30 cm in diameter, ca. 12 cm in water-depth, 28°–30°C) with continuous lighting. Both the fry and the adults were fed a powdered diet [9]. They were inbred by sister-brother matings for six generations during a period of two years from 1989 to 1991.

For observations by transmission electron microscopy, previtellogenic follicles in ovarian tissue were fixed with modified Karnovsky's fixative for more than 6 hr at 4°C. After the samples were rinsed, post-fixed for 60 min in phosphate-buffered (pH 7.2) 1% OsO_4 at 0–4°C, and dehydrated in an alcohol and acetone series, they were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JOEL JEM-100C electron microscope.

The data were statistically analysed by the Student's t-test.

RESULTS

The spiral pattern of attaching and non-attaching filaments in various sized oocytes during oogenesis

Primitive attaching and non-attaching filaments were first recognized as verruciform structures in the space between the surface of transparent small oocytes (about 120 μm in diameter: Stage IV in 7) and the surrounding follicular cells just after the formation of very thin rudiments of chorion (Fig. 1). Most of the verruciform structures were distributed at intervals of about 12.5 μm on the chorionic surface in St. IV oocytes [7]. The primitive attaching filaments, which were distributed at intervals of about 8 μm, were clustered in the vegetal pole area (VPA). The non-attaching filaments first became horn-shaped (Fig. 1), then candle-like (Fig. 2), which caused them to bend in the same direction in St. IV oocytes (130–140 μm in diameter). They bent at right angles to the oocyte axis, one end of which was determined by the VPA with its verruciform structures (primitive attaching filaments). Attaching filaments situated at the VPA of the oocyte became unilaterally curved candle-like structures in St. IV oocytes 140–150 μm in diameter (Fig. 2, Table 1). When the oocyte diameter reached about 350 μm (St. VI), the mean distance between adjacent attaching filaments was about 14 μm (the diameter of the VPA, ca. 160 μm) which represented an increase of only 5 μm per 100 μm increase in oocyte diameter. In contrast, the interval between non-attaching filaments was about 23 μm and increased about 8 μm per 100 μm increase in oocyte diameter (Fig. 3). As the
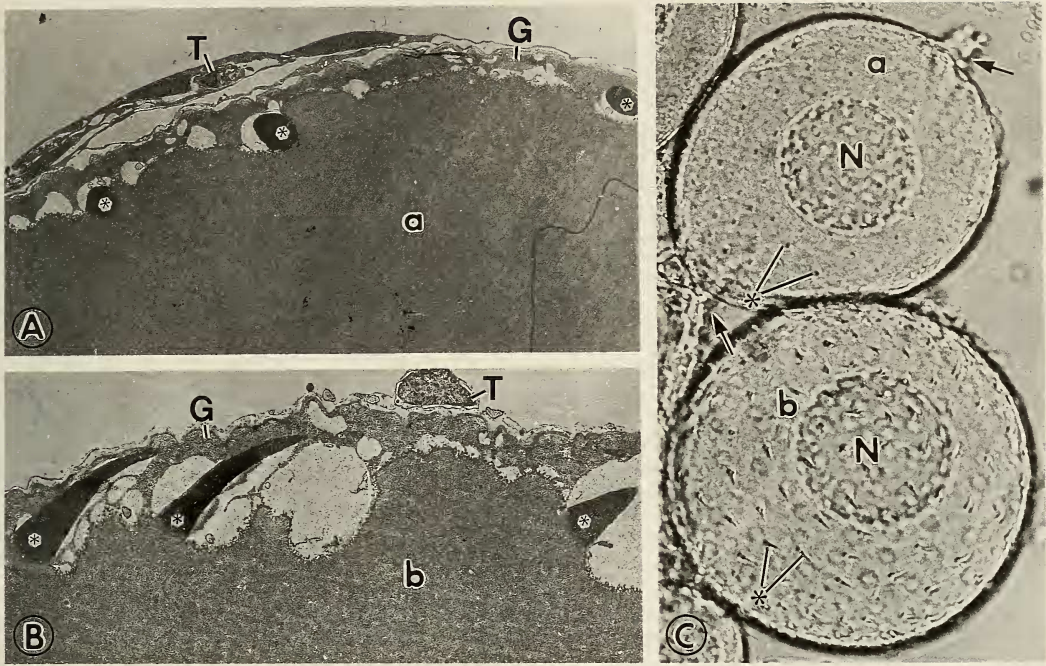
FIG. 1.   Early previtellogenic oocytes showing verruciform or horn-shaped non-attaching filaments on the oocyte surface in *Oryzias latipes*.

A: Electron micrograph of a part of an oocyte (a) with rudiments non-attaching filaments present as verruciform structures (asterisks). ×2,100.

B: Electron micrograph of a part of an oocyte (b) with horn-shaped nonattaching filaments (asterisks). ×2,700

C: Micrograph of intact intrafollicular previtellogenic oocytes showing verruciform filaments as dots (asterisk in oocyte a) and horn- or stickle-shaped non-attaching filaments (asterisk in oocyte b). These oocytes correspond to those in Figs. A and B, respectively. Arrows indicate fibrous structures connecting with the other ovarian tissues. G, granulosa cell layer; N, oocyte nucleus; T, thecal cell layer. ×415.

vitellogenic oocytes grew rapidly, the surface of the chorion expanded accordingly except for the VPA which expanded only slightly and occupied an area 410–450 μm in diameter in mature oocytes.

Attaching and non-attaching filaments arose from the chorion and elongated among the granulosa cells within the basement membrane (Fig. 4A). These filaments were composed of bundles of tubular structures (18–19 nm outside diameter), as reported by Hart and Donovan [10]. They arose from and morphologically resembled the outer layer of the chorion, which also consisted of electron-dense and microtubular structures (16–18 nm outer diameter) (Fig. 4B).   The numbers of attaching and non-attaching filaments on an oocyte of *O. latipes* (25–35 and 190, respectively) did not change regardless of the size of the growing

oocytes. The attaching filaments clustered at the vegetal pole of the oocyte axis elongated in a unilateral direction around the oocyte axis. Long attaching filaments, which began to form in previtellogenic oocytes eventually formed a special cap over the vegetal hemisphere as they elongated progressively in the vitellogenic oocyte (Figs. 2 and 5).   As shown in Table 1, both left- and right-handed spiral patterns of attaching filaments (Fig. 6) were present.

Inheritance of the spiral pattern of attaching filaments of O. latipes eggs

Fifteen fertilized eggs (F_0) showing the right-handed spiral pattern of attaching filaments were selected from a batch of eggs spawned by a single pair (sister-brother mating) of orange-red type *O. latipes* and developed to adulthood. Three pairs
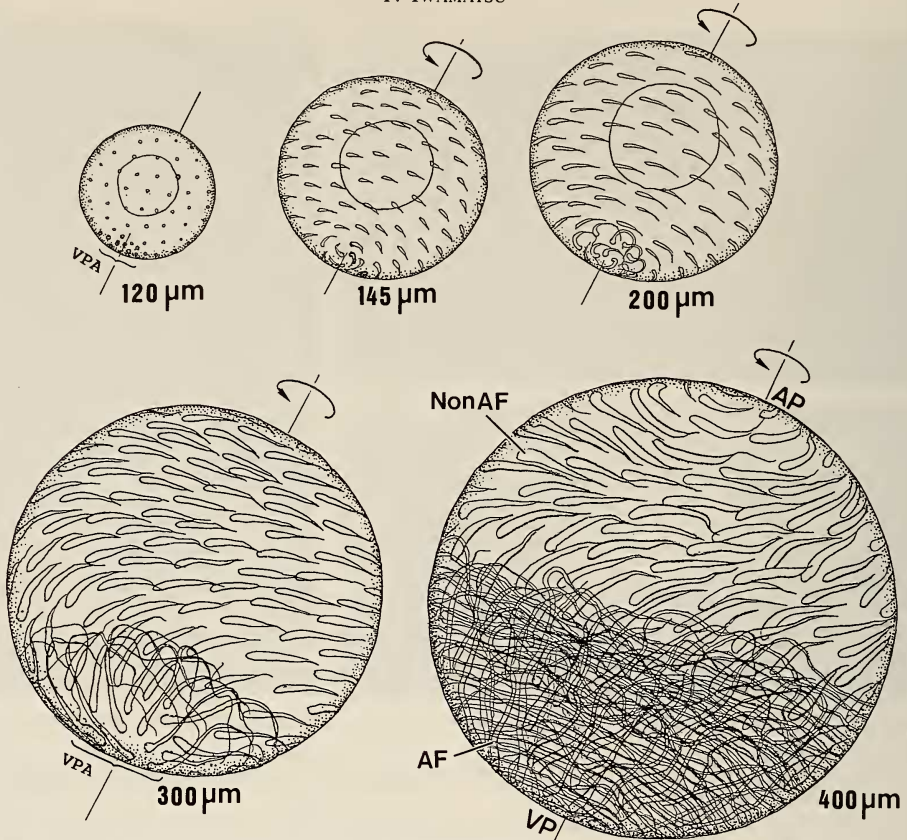
FIG. 2. A diagram of the changes in attaching and non-attaching filaments during oocyte growth in *Oryzias latipes*. This diagram displays the successive changes in non-attaching (Non-AF) and attaching (AF) filaments (the left-handed spiral type) of growing oocytes. The filaments bend in a unilateral direction around the animal (AP)-vegetal (VP) axis (oblique bars). Attaching filaments (AF) are spun over the chorion in the vegetal hemisphere of a large oocyte (400 μm). A presumptive direction (the right-handed) of the rotation of oocytes is indicated by an arrow.

TABLE 1. Differentiation of filaments on the chorion and the left- or right-handed spiral pattern of the filaments in *Oryzias latipes* eggs

Size of oocytes (μm)	No. of oocytes (Females)	Spiral pattern of filaments (%)*		Attaching filaments (%)*	Non-attaching filaments (%)*		
		Right	Left		V	H	B
<110	56	—	—	ND	ND	ND	—
110–119	3	—	—	ND	100	0	0
120–139	44	—	—	ND	54	39	7
140–149	41	36	64	27	0	17	83
150–199	116	45	55	100	0	0	100
200–299	121	46	54	100	0	0	100
300–399	51	63	37	100	0	0	100
400–499	33	70	30	100	0	0	100
500–600	16	44	56	100	0	0	100
>600	65	58	42	100	0	0	100

* Percentages of the number of oocytes showing attaching filaments or non-attaching filaments among the total number of oocytes in each size. Abbreviations for non-attaching filaments: B, unilaterally bended; H, short horn-shaped; V, verruciform.
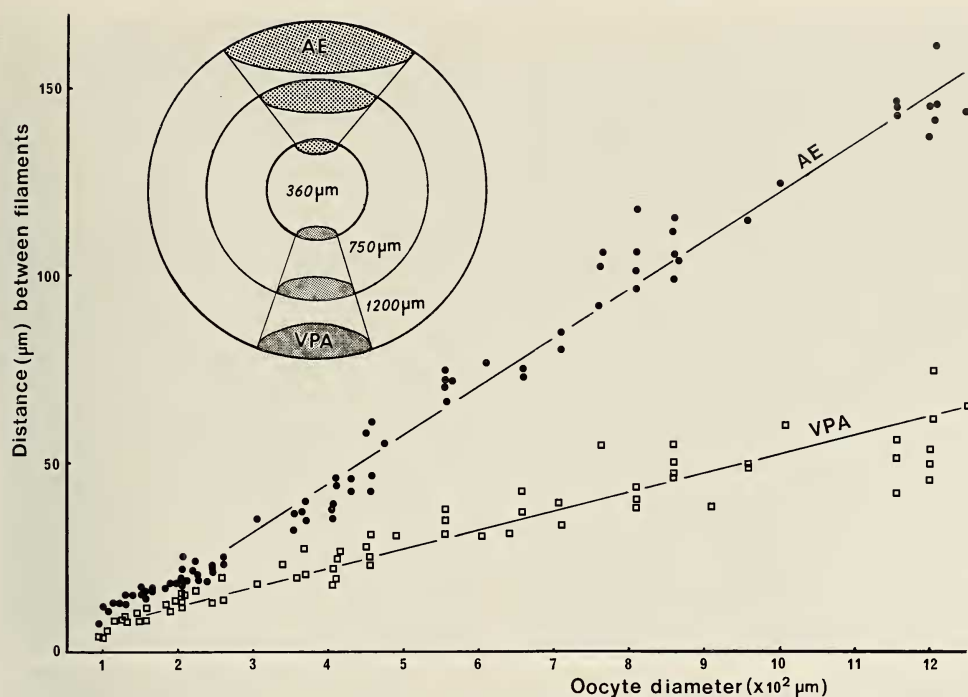
FIG. 3.   Change in the distance between filaments during oocyte growth.   The change in the distance between adjacent filaments on the chorion in proportion to the increase in the oocyte diameter (μm) is diagramed at the upper left.   The mean distances between filaments in the VPA (open squares) and in the remainer of the chorion surface (AE: the animal pole and the equatorial area, closed circles) was spotted against oocyte diameter.

selected at random from among the sexually mature fish were mated yielding 218 fertilized eggs in 16 ovipositions.   Among these spawned eggs (F_1), about 64% (a right-handed to left-handed type (R/L) ratio, 1.6) showed the right-handed spiral pattern of attaching filaments.   Six pairs that developed from these right-handed type eggs were selected again for mating (each pair was mated in a separate aquarium).   A total of 623 eggs was obtained in 41 ovipositions.   The total R/L ratio among these F_2 eggs was 1.1, although the number of right-handed type eggs among these eggs of two pairs (FR-F_{2-1}, FR-F_{2-2}) was significantly higher. The F_3 (FR-F_{3-15}) eggs spawned by the FR-F_{2-1} pair in Table 2 were reared and again pair-mated. The right-handed spiral pattern among the F_4 (FR-F_{4-19}) eggs (1625) spawned by these pairs (FR-F_{3-4}) was not significantly more prevelant than the left-handed pattern.   Two pairs (FR-F_{5-13}) of the off-spring of FR-F_{4-5} spawned 604 eggs (F_6), which showd a R/L ratio of 1.0.   In the

right-handed type pedigrees, the R/L ratio among 5,002 eggs in six generations was about 1.1.

The spiral pattern of attaching filaments in several species of Oryzias

Table 3 lists the R/L ratios of the spiral pattern of attaching filaments on the chorions of *O. celebensis*, *O. curvinotus*, *O. javanicus*, *O. mekongensis*, *O. melastigma* and *O. minutilus* eggs.   The right-handed pattern tended to occur more frequently than the left-handed pattern, but the difference was not statistically significant.

DISCUSSION

Both the left- and the right-handed spiral patterns are found in the attaching filaments on the chorion of the medaka egg.   A similar observation on the spiral pattern of filamentous structures on the chorion was reported early by Kurakami [11] for the pacific saury *Cololabais saira*.   In a pre-
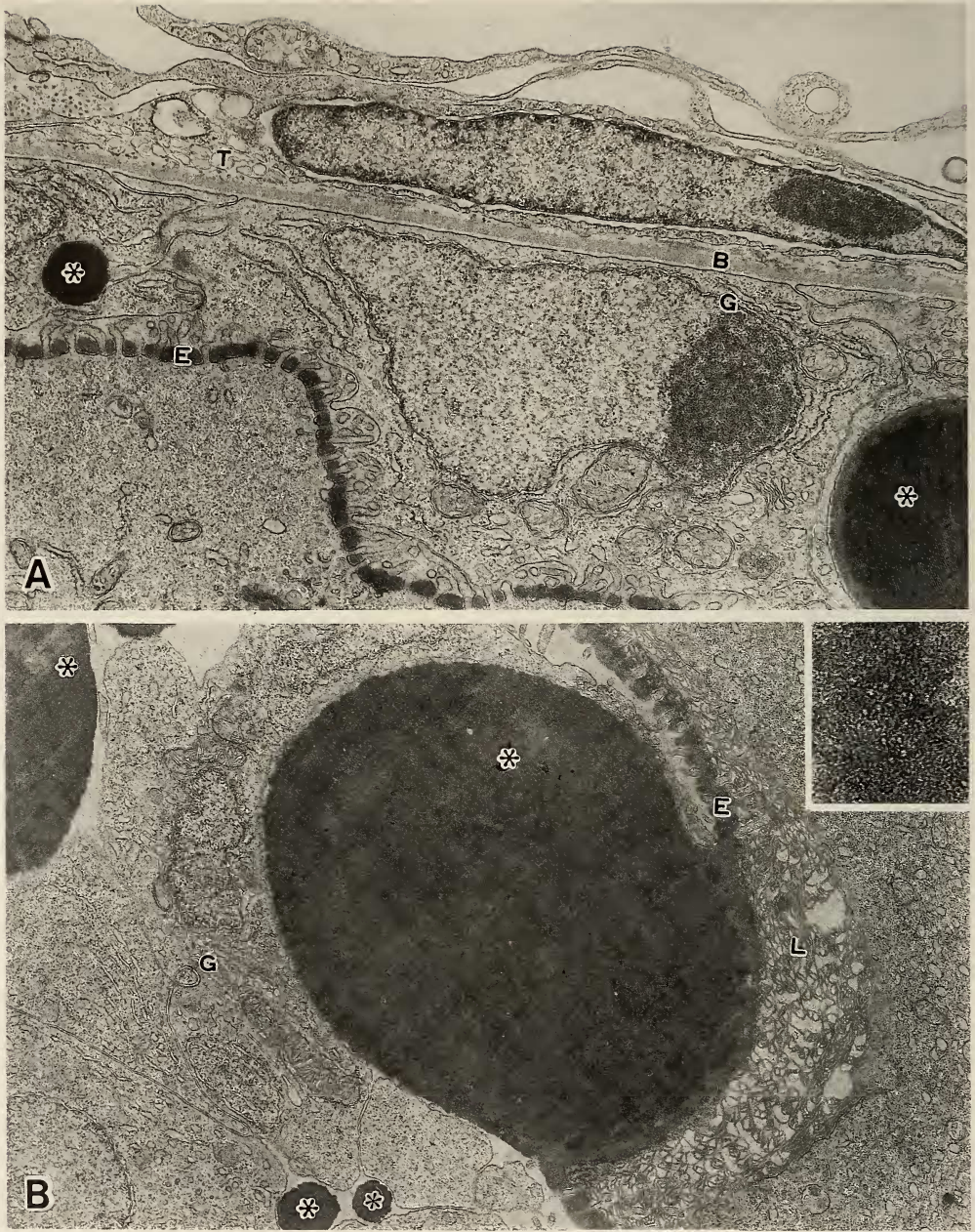
FIG. 4.   Micrographs of previtellogenic oocytes showing non-attaching filaments among granulosa cells.
A: An oocyte at the late previtellogenic phase of oogenesis exhibits non-attacing filaments (asterisks) among the granulosa cells (G) between the chorion (E) and the basement membrane (B). T, thecal cell. ×13,000.
B: An oocyte at an early vitellogenic phase exhibits non-attaching filaments (asterisks) originating from the chorion, which consists of an outer layer (E) and an inner layer (L). G, granulosa cells. ×9,700. Inset: the outer layer of the chorion which is composed of electron dense amorphous and tubular structures. ×92,000.
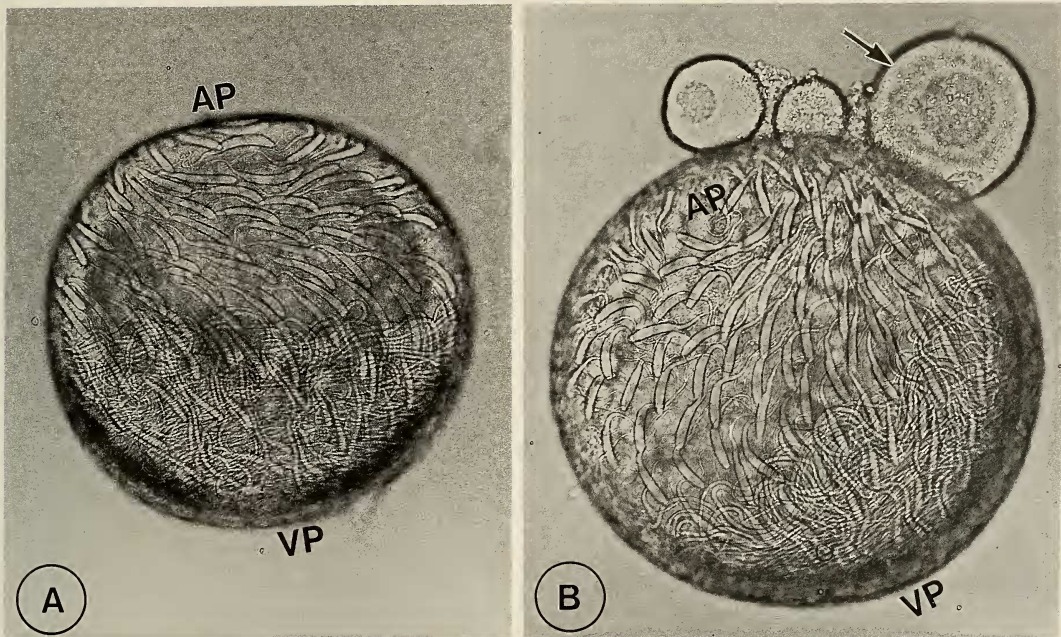
FIG. 5. Microphotographs of vitellogenic oocytes of *Oryzias latipes* showing attaching filaments on the chorion in the vegetal hemisphere. These oocytes at the early stage (A: the right-handed spiral type) and at the late stage (B: the left-handed spiral type) of vitellogenesis have different spiral patterns. Non-attaching filaments in the animal hemisphere bend and the thread-like attaching filaments elongating from the VPA are wound on the vegetal hemisphere of the oocyte in a unilateral direction. The proximal part of the attaching filaments bends in the direction of the animal pole of the oocyte. In Fig. 5B, early previtellogenic oocytes (ca. 115 μm in diameter, arrow) are present showing verruciform primitive filaments. $\times$210.

liminary note [8], It has been reported that eggs showing the right-handed spiral of attaching filaments might be counted more frequently than those showing the left-handed spiral. This tendency was also recognized in eggs of other *Oryzias* species in the present study, although the higher frequency was not statistically significant, and eggs spawned by only a single female of each species were studied.

In addition, we tried to select genetically for the eggs showing the right-handed spiral pattern of attaching filaments. The results seem to indicate that the spiral pattern is not hereditory. That is, the spiral pattern may be randomly determined during oogenesis. The tendency for the right-handed type to somewhat predominant in frequency is possibly caused by some unknown factor(s) such as the revolution of the earth.

The spiral of attaching filaments and the un-ilateral bending of non-attaching filaments on the chorion are both at right angles to the oocyte axis and appear to be involved in the formation of egg polarity. All filaments must elongate in haphazard and random directions among the granulosa cells if no force is exerted on them. However, since all elongating filaments arise from the chorion and elongate among the granulosa cells, they should bend in the same direction if the oocyte itself rotates within the non-motile follicular consti-tuents connected to the other tissue of ovary, or if all the granulosa cells translocate in a unilateral direction in response to unknown stimulation. Granulosa cells directly contact to the oocyte surface by inserting their cytoplasmic extensions into the chorion, and the cytoplasmic extensions of the oocyte also closely attach to the granulosa cells through numerous pores in the chorion until ovula-tion [12]. For this reason, it is unlikely that either translocation of the granulosa cells or the rotation of the oocyte within the non-motile granulosa cell
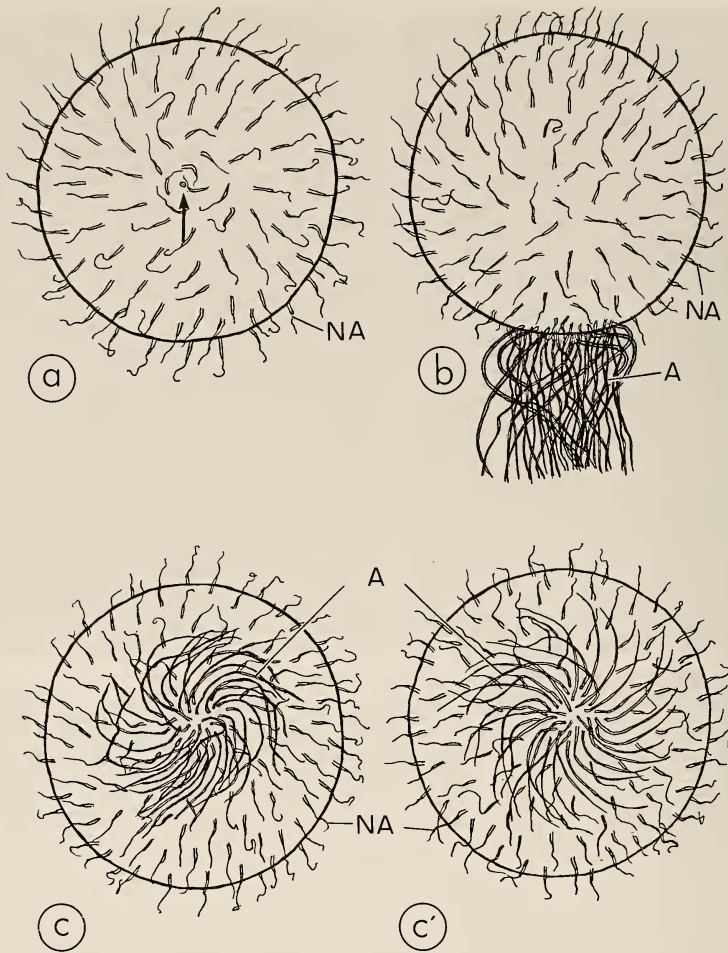
Fig. 6.   Diagrams illustrating the patterns of attaching and non-attaching filaments on the chorion of mature *Oryzias* eggs.
a: A view of the animal pole side (Arrow indicates a micropyle: non-attaching filaments, the right-handed pattern).
b: A lateral view from the equatorial side. c and c': In these views of the vegetal pole side, attaching filaments (A) exhibit either the left-handed (c) or the right-handed (c') spiral pattern. NA, non-attaching filaments.

layer can occur because both would have to be accompanied by mutual separation of the oocyte and granulosa cells before ovulation. It is plausible that the oocyte intimately connected with its surrouding granulosa cells can translocate within the basement membrane. In young previtellogenic oocytes, the granulosa cell layer is extremely thin, so the tops of the elongating filaments are compressed by the basement membrane and the thecal layer.   Consequently, unilateral rotation of the oocyte and the surrounding granulosa cell layer within the basement membrane may cause un-ilateral bending at the distal part of the elongating filaments on the chorion. Rotation of the oocyte must result in bending of only the attaching and non-attaching filaments that are directly in contact with the basement membrane, and the bending must be in the opposite direction to that of the rotation. The basal regions of attaching filaments, which are surrounded by tall granulosa cells, and the long basal parts of attaching filaments in the vegetal hemisphere are not affected by the oocyte rotation and are radially straight from the VPA toward the animal pole after slightly bending dur-

TABLE 2. Inheritance of the right-handed spiral pattern of attaching filaments in *Oryzias* eggs

| Parent | Date | No. of ovipositions | No. of eggs | Spiral pattern of attaching filaments | | R/L ratio |
				Right-handed (L)	Left-handed (R)	
FR-F_0	May '90	1	25[#]	13	12	1.0
FR-F_1	Aug. '90	16	218[#]	140	78	1.6*
FR-F_{2-1}	Oct. '90	5	54[#]	35	19	1.8*
FR-F_{2-2}	″	7	95	54	41	1.3*
FR-F_{2-3}	″	6	113	57	56	1.0
FR-F_{2-4}	″	16	209	98	111	0.9
FR-F_{2-5}	″	4	73	40	33	1.2
FR-F_{2-6}	″	3	79	38	41	0.9
FR-F_{3-1}	Feb. '91	16	439	229	210	1.1
FR-F_{3-2}	″	22	471	251	220	1.1
FR-F_{3-3}	″	14	364(145)	185(72)	179(73)	1.0
FR-F_{3-4}	″	16	417(117)[#]	224(66)	193(51)	1.2
FR-F_{3-5}	″	11	215 (172)	121(74)	94(98)	1.3
FR-F_{4-1}	Aug. '91	10	257	132	125	1.1
FR-F_{4-2}	″	10	195	91	104	0.9
FR-F_{4-3}	″	10	159(103)	74(47)	85(56)	0.9
FR-F_{4-4}	″	11	121(87)	63(47)	58(40)	1.1
FR-F_{4-5}	″	18	224[#]	126	98	1.3
FR-F_{4-6}	″	16	284	132	152	0.9
FR-F_{4-7}	″	12	163	96	67	1.4
FR-F_{4-8}	″	13	139	72	67	1.1
FR-F_{4-9}	″	12	84	41	43	1.0
FR-F_{5-1}	Oct. '91	8	244	130	114	1.1
FR-F_{5-2}	″	8	142	66	76	0.9
FR-F_{5-3}	″	11	218	112	106	1.1

P/L ratio: the ratio of the number of eggs with the right-handed spiral pattern of attaching filaments to the nubmer with the left-handed spiral pattern. F3 were obtained from a single pair of FR-F_{2-3} adults. *Significant in Student's t-Test. [#] Mature fish derived from these eggs were used for mating. The number in parentheses indicates the number of oocytes in ovaries.

TABLE 3. The spiral pattern of attaching filaments in eggs of several species of *Oryzias*

| Species | No. of ovipositions | No. of eggs | Spiral pattern of attaching filaments | | R/L ratio |
			Right-handed (R)	Left-handed (L)	
O. celebensis	13	340	188	152	1.2
O. curvinotus	2	82	48	34	1.4
O. javanicus	10	55	35	20	1.8
O. mekongensis	5	93	53	40	1.3
O. melastigma	27	480	287	193	1.5
O. minutilus	6	69	39	30	1.3

ing the early stage of oogenesis (Fig. 3). Thus, it is inferred that in medaka follicles, oocytes surrounded by a granulosa cell layer may begin to rotate within the basement membrane before or when the oocyte axis is established in the very early stage of oogenesis, although the transloca-

tion of the granulosa cell layer within the basement membrane remains to be investigated.

Associated with the rotation of the oocyte and granulosa cells on the oocyte axis, the first morphological evidence of *Oryzias* egg polarity is the appearance of the VPA. The VPA appears as cluster of verruciform structures, which are primordia for the attaching filaments, prior to formation of the chorion. As these filaments become progressively horn-shaped, they bend in a unilateral direction with the rotation of the oocyte around its axis. This axis corresponds to the future animal-vegetal axis of the egg prior to differentiation of the long, slim attaching filaments at the VPA and the micropylar cell at the animal pole [1]. The oocyte axis may be established by an unknown factor(s) that determines the position of the VPA. Investigations on determination of the origin and the formation process of these filamentous structures on the chorion may contribute to clarifying the determination of egg polarity in some fishes. However, it remains to be determined whether these filaments, as well as the chorion, are derived from the oocyte itself [13], the follicle cells or both [10, 14].

As described above, the animal-vegetal axis of *Oryzias* eggs is first recognized by the appearance of the VPA and the unilateral bending of non-attaching filaments prior to the initiation of vitellogenesis. Therefore, the egg axis is not induced by an unequal distribution of the yolk that accumulates in the ooplasm during vitellogenesis. These observations with *Oryzias* oocytes are consistent with those with *Xenopus* oocytes, which may have an animal-vegetal polarity that is further differentiated by the endocytotic process for vitellogenin (15). In addition to yolk accumulation, a change in the distribution of attaching and non-attaching filaments on the chorion is recognized as the vitellogenic oocyte grows rapidly. The interfilament distance between non-attaching filaments on the chorion increases in proportion to the increase in diameter of vitellogenic oocytes, in contrast to the distance between attaching filaments on the chorion in the VPA. This suggests that the outer layer of the chorion is stretched except in the VPA where the attaching filaments elongate extremely during vitellogenesis. Attaching filaments consist

of the same components as the chorion [10] or the outer layer of the chorion as shown in the present study. Therefore, in the chorion of the VPA the components which are supplied for formation of the outer layer probably by follicular cells [4, 10] should be limited due to their use in formation of the elongating attaching filaments. Consequently, less chorion is formed in the VPA than in the remaining area of the chorion. That is, since there is no change in the total number of filaments on the chorion, the lesser distance between attaching filaments must result from a lesser expansion of the chorion in the VPA than in the remaining area of the chorion. Further experimentation is being conducted currently in order to analyse the process of formation of the VPA and the mechanism responsible for the spiral pattern of attaching filaments in this fish.

ACKNOWLEDGEMENTS

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